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## Identification and Characterization of Two Novel Clostridial Bacteriocins, Circularin A and Closticin 574

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**Two novel antibacterial peptides of clostridial species were purified, N-terminally sequenced, and characterized. Moreover, their structural genes were identified. Closticin 574 is an 82-amino-acid bacteriocin produced by *Clostridium tyrobutyricum* ADRIAT 932. The supernatant of the producing strain showed a high level of activity against the indicator strain *C. tyrobutyricum*. The protein is synthesized as a preproprotein that is possibly secreted via the general secretion pathway, after which it is hydrolyzed at an Asp-Pro site. Circularin A is produced by *Clostridium beijerinckii* ATCC 25752 as a prepeptide of 72 amino acids. Cleavage of the prepeptide between the third leucine and fourth valine residues followed by a head-to-tail ligation between the N and C termini creates a circular antimicrobial peptide of 69 amino acids. The unusually small circularin A leader peptide of three amino acids is cleaved off in this process. The supernatant of *C. beijerinckii* ATCC 25752 showed a broad antibacterial activity range.**

Clostridia have a negative image as many species are known as pathogens (50), toxin producers (50), and food-spoilage bacteria (36). Various clostridia produce bacteriocins, but despite their long history as a means of typing clostridia (33, 39), only little sequence, functional, or structural information is available on these antibacterial peptides. The few clostridial bacteriocins that have been further characterized are BCN5 and boticin B (12, 18). BCN5 is a large (97-kDa) UV-inducible protein produced by *Clostridium perfringens* (18). Boticin B is produced by *Clostridium botulinum* as a small peptide with a predicted size of 50 amino acid residues (12). Little is known about the regulation of production or secretion of these bacteriocins. Generally, bacteriocins are small ribosomally synthesized antimicrobial peptides (27, 35). They are mostly membrane permeabilizing and cationic, and they typically comprise fewer than 50 amino acid residues (35). Bacteriocins can be used as an additive to food products to prevent the growth of spoilage bacteria (10). Klaenhammer divided bacteriocins into four classes (35). Class I bacteriocins, known as lantibiotics, contain posttranslationally modified residues such as lanthionine,  $\beta$ -methyl lanthionine, and dehydrated residues. Class II bacteriocins lack these modifications and are linear peptides (35, 44). Class III contains the large, heat-labile bacteriocins. Class IV bacteriocins are complex molecules composed of protein and chemical moieties. Both class I and class II bacteriocins have been intensively studied, and more than 20 and 100 representatives are known, respectively.

The subclass IIc (class II bacteriocins other than the pediocin-like bacteriocins or the two-peptide bacteriocins) contains a few examples of bacteriocins that are produced as circular molecules. This circularization is the result of a head-to-tail

peptide bond formation of a prepeptide. Bacteriocins with this typical structure are microcin J25, gassericin A, and AS-48/Bac21 (6, 31, 40, 59). Microcin J25 is produced by *Escherichia coli* as a 58-amino-acid precursor which, after processing, yields a cyclic peptide of 21 amino acid residues. Two proteins are involved in the maturation of microcin J25 while an ABC transporter homologue is involved in transport of and immunity to the bacteriocin (56). Gassericin A is produced by *Lactobacillus gasseri* as a peptide of 58 amino acid residues with a 33-amino-acid leader. Nothing is known of the maturation or secretion process of this bacteriocin (32). The *Enterococcus faecalis* AS-48 (*bac21*) primary translation product of 116 amino acid residues is processed to create a cyclic bacteriocin of 70 amino acids. Four gene products (AS-48B, AS-48C, AS-48C1, AS-48D) are involved in the production of AS-48. A fifth gene product (AS-48D1) confers immunity to the bacteriocin (41). AS-48 has a three-dimensional structure showing homology to that of NK-lysin of porcine T cells (22). NK-lysin is a noncircular protein of 78 amino acid residues that has antibacterial and tumolytic activities (4).

Other cyclic antibacterial peptides exist that are nonribosomally synthesized, like mycosubtilin, gramicidin-S, and tyrocidine (11, 13, 43). These peptides can contain, besides L-amino acids, amino acids in the D configuration and uncommon amino acids such as ornithine (43).

As a first step towards preventing the growth of *Clostridium tyrobutyricum*, a known food spoiler (36), we sought to identify bacteriocins active against this bacterium. As the activity range of many bacteriocins is limited to closely related species, we screened clostridia for production of anti-*C. tyrobutyricum* activities.

In this article, we describe the purification of two novel antibacterial peptides produced by two different clostridial species. Closticin B574 is produced by *C. tyrobutyricum* ADRIAT 932 and belongs to the class II bacteriocins. Circularin A, produced by *Clostridium beijerinckii* ATCC 25752, is shown to

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TABLE 1. Bacterial strains

Strain	Description	Reference or source <sup>a</sup>
<i>C. beijerinckii</i> ATCC 25752	Circularin A producer	NIZO
<i>C. tyrobutyricum</i> NIZO B570		NIZO
<i>C. tyrobutyricum</i> ADRIAT 932	Clostin 574 producer	NIZO
<i>C. tyrobutyricum</i> NIZO B575		NIZO
<i>C. tyrobutyricum</i> CNRZ500		NIZO
<i>C. tyrobutyricum</i> NIZO B590		NIZO
<i>E. faecalis</i> JH2-2		28
<i>E. faecalis</i> OG1X		25
<i>Lactobacillus alimentarius</i> L4		Unilever
<i>Lactobacillus brevis</i> L40		Unilever
<i>Lactobacillus buchneri</i> L4		Unilever
<i>Lactobacillus casei</i> subsp. <i>casei</i> L37		Unilever
<i>Lactobacillus plantarum</i> ATCC 14917		Unilever
<i>L. sakei</i> ATCC 15521		Lab collection
<i>L. sakei</i> IFO12456		Lab collection
<i>L. lactis</i> IL1403	Plasmid free	8
<i>L. lactis</i> MG1363	Plasmid free	19
<i>Pediococcus pentosaceus</i> FBB61-2		Lab collection
<i>Pediococcus pentosaceus</i> PPE 1.2		49

<sup>a</sup> NIZO, NIZO food research (Ede, The Netherlands). Unilever is located in Vlaardingen, The Netherlands.

be a cyclic antibacterial peptide. The bacteriocins were purified from culture supernatants of the respective producer strains, and then the structural genes were cloned via reverse genetics techniques.

#### MATERIALS AND METHODS

**Bacterial strains, media, and reagents.** The strains and plasmids used in this study are listed in Table 1. Clostridia were grown anaerobically at 30°C in reinforced *Clostridium* medium (RCM) (Merck, Darmstadt, Germany) or in AC broth (Difco, Detroit, Mich.) for bacteriocin production. Cultures were grown anaerobically by flushing with oxygen-depleted nitrogen or by chemical absorption of the oxygen. Medium was heated for 5 min at 120°C to evacuate gasses and chilled on ice. After inoculation, a cotton cloth with an equal volume of 10% pyrogallol (MERCK-Schuchardt, Hohenbrunn, Germany) and saturated sodium carbonate was applied and the tube was sealed airtight. Plates were incubated in anaerobic jars with an anaerocult A sachet (Merck). *Lactobacilli* and *pediococci* were grown in MRS at 30 and 37°C, respectively. Twofold-diluted M17 (Difco) with a final concentration of 1.9%  $\beta$ -glycerophosphate and 0.5% glucose (G[1/2]M17) was used for growth of *Listeria innocua* and *Lactococcus* sp. *E. coli* was grown in TY broth at 37°C with vigorous agitation. The appropriate media were supplemented with 1.5% agar for growth of bacteria on agar plates. Ampicillin was used at a concentration of 100  $\mu$ g/ml, and chloramphenicol and erythromycin were used in combination at concentrations of 2.5  $\mu$ g/ml each.

**General DNA methods and materials.** Molecular cloning techniques were performed essentially as described by Sambrook et al. (52). Restriction enzymes, T4 DNA ligase, and Expand DNA polymerase were obtained from Roche Diagnostics GmbH (Mannheim, Germany) and used as specified by the supplier. Plasmids were isolated from clostridial strains according to the method of Anderson et al. (2).

**Tricine-SDS-PAGE.** Proteins were separated by Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (53) with 20% acrylamide (Bio-Rad, Hercules, Calif.). Protein samples were mixed with an equal

volume of 2 $\times$  Laemmli sample buffer (100 mM Tris [pH 6.0], 20% glycerol, 0.1% bromophenol blue [Merck]) with or without 4% SDS and 10%  $\beta$ -mercaptoethanol. The low-range rainbow marker RPN 755 (Amersham, Roosendaal, The Netherlands) was used as a protein molecular weight marker. Silver staining was performed according to the protocol of Wray et al. (62).

**Bacteriocin assays.** Colony overlay assays were performed. Colonies on a nutrient plate were treated with chloroform vapor for 15 min. After 30 min of exposure to air, the colonies were covered with a top agar (0.75%) layer of the proper growth medium seeded with the indicator strain. After appropriate incubation, 1 to 3 days depending on the indicator strain used, the plates were examined for halos of growth inhibition. Bacteriocin activity was determined by a critical dilution assay as described by Geis et al. (20), except that assays were performed in microtiter plates. To 50  $\mu$ l of serial-diluted bacteriocin-containing samples, 150  $\mu$ l of medium containing the indicator strain *Lactobacillus sakei* ATCC 15521 (pMG36e, pMG36c) was added, unless mentioned otherwise. The volumes were 100  $\mu$ l for both sample and indicator when clostridia were used as indicators. After the appropriate incubation time (16 to 24 h for *L. sakei* or 3 days for the clostridia), the microtiter plates were examined for growth inhibition. Inhibition is seen when the optical density at 600 nm in a well is less than half of that of a control well without added bacteriocin. The highest dilution of bacteriocin still inhibiting growth of the indicator is set to contain 1 arbitrary unit (AU). Bacteriocin production during growth was determined by growing the producer anaerobically in AC medium in krim seal bottles in a nitrogen atmosphere. Samples were taken by syringe at regular time points without disturbing the anaerobicity. To determine bacteriocin activity in a Tricine-SDS-polyacrylamide gel, the procedure of Bhunia et al. (7) was used with the following modifications. Fixation of the gel was done by incubation in 20% isopropanol-10% acetic acid in water for 30 min at room temperature. Subsequently, the gel was washed six times with demineralized water, covered with a top agar (0.75%) layer containing 10<sup>-2</sup> diluted cells of an overnight culture of the indicator *L. sakei*, and incubated overnight at 30°C.

**Bacteriocin characterization.** Screening for bacteriocin production by clostridia was performed by measuring the antibacterial activity of filter-sterilized supernatants of stationary-phase cultures grown in RCM basically as described previously (20), except that the experiments were performed in 200- $\mu$ l final volumes in microtiter plate wells. Microtiter plates were incubated anaerobically for 3 days before inhibition levels were determined. Additionally, bacteriocin production on plates was tested by a colony overlay assay. Six clostridial strains were used as indicators. The proteinase sensitivity of putative bacteriocin activities was determined by incubating filter-sterilized supernatants of stationary-phase cultures of bacteriocin-producing strains for 1 h (clostin 574) or 24 h (circularin A) at 37°C with an equal volume of 1.0 mg of proteinase K (Merck), thermolysin, alpha-chymotrypsin (Sigma, St. Louis, Mo.), or pronase (Roche) per ml or 0.78 mg of trypsin (Sigma)/ml in 0.1 M Tris buffer (pH = 7.6). The residual bacteriocin activity was determined in a critical dilution assay. Bacteriocin temperature stability was determined by incubating filter-sterilized supernatants for 1 h at temperatures ranging from 4 to 90°C. After incubation, samples were cooled on ice and residual activity was measured in a critical dilution assay. Antibacterial activity as a result of the presence of a phage in the culture supernatant was excluded by filtering the culture supernatants through a 0.2- $\mu$ m-pore-size filter (Schleicher & Schuell, Dassel, Germany) and testing the filtrate for antibacterial activity. The requirement of disulfide bridges for bacteriocin activity was determined by measuring the activity in a critical dilution assay in the presence of 10 mM dithiothreitol (DTT). The bacteriocin activity range was determined in a microtiter plate assay. Two hundred microliters of an exponentially growing culture of an indicator strain, diluted either 1:500 or 1:1,000 in fresh medium, was added to 50  $\mu$ l of bacteriocin-containing supernatant. Plates were incubated at the appropriate temperature for 18 h, and growth was determined by measuring the optical cell density at 600 nm in a microtiter plate reader (Tecan, Männedorf, Switzerland).

**Bacteriocin purification.** For the purification of circularin A, *C. beijerinckii* ATCC 25752 was grown on an AC medium dialysate. The dialysate was obtained by dialyzing 100 ml of 10 $\times$  concentrated medium against 900 ml of water by using a dialysis membrane (Medicell, London, England) with a cutoff of 12 to 14 kDa. The supernatant of a filter-sterilized culture was precipitated with 50% ammonium sulfate at 4°C for at least 4 h. The precipitate was spun down at 12,000  $\times$  g for 30 min at 4°C and dissolved in 30 ml of demineralized water. This solution was extracted with an equal volume of *n*-butanol. The *n*-butanol phase was freeze-dried, and the resulting pellet was again dissolved in water. This fraction was further purified by high-performance liquid chromatography (HPLC) with a C<sub>4</sub> column and eluted with a 10 to 90% acetonitrile gradient in a 0.1 to 0.05% trifluoroacetic acid gradient at 1 ml/min while the absorbance at 214 nm was

continuously monitored. The active, bacteriocin-containing fraction after HPLC was used for N-terminal amino acid sequence determination.

Clostin 574 was purified from the supernatant of a stationary-phase culture of *C. tyrobutyricum* ADRIAT 932 grown in AC medium. The culture supernatant was filter sterilized, and proteins were precipitated with 40% ammonium sulfate for at least 4 h at 4°C. The precipitate was spun down at  $12,000 \times g$  for 30 min and dissolved in 20 ml of demineralized water. This fraction was further purified with a hydrophobic interaction column (Octyl Sepharose Fast Flow; Amersham) and fast-performance liquid chromatography (FPLC). The column was washed with 1.0 M ammonium sulfate in a 50 mM phosphate buffer (pH 7.0) containing 30% isopropanol. Bacteriocin activity was eluted with a 1.0 to 0 M ammonium sulfate gradient in 50 mM phosphate buffer (pH 7.0) containing 30% isopropanol at 1 ml/min. Absorbance was monitored at 280 nm. The purified material was desalted and concentrated with an Ultrafree-4 ultrafiltration column with a 5-kDa cutoff (Millipore, Bedford, Mass.). The retentate was applied to an anion-exchange column (DEAE Sepharose fast flow; Amersham), washed with 20 mM Tris-HCl (pH 8.0), and eluted with a 0 to 2.0 M NaCl gradient in 20 mM Tris-HCl (pH 8.0). The flowthrough was concentrated with an Ultrafree-4 ultrafiltration column with a 5-kDa cutoff (Millipore). Proteins were further purified by HPLC with a  $C_4$  column, which was eluted with a 10 to 90% acetonitrile gradient in 0.1% trifluoroacetic acid at 1 ml/min. Absorbance was monitored at 214 nm. Protein purity and quantity were determined by Tricine-SDS-PAGE and subsequent silver staining.

**N-terminal sequence determination.** N-terminal amino acid sequencing was performed at Eurosequence bv (Groningen, The Netherlands). Sequence analysis was performed by Edman degradation (14, 26) with an automated sequenator (model 494 Procise or model 477a; Applied Biosystems, Warrington, United Kingdom) (24) and protocols, reagents, chemicals, and materials from Applied Biosystems.

**Nucleotide sequencing.** Degenerate primers were designed based on the amino acid sequences of the internal peptides fragment A and fragment B of circularin A and on the N-terminal amino acid sequence of clostin B574. Two primers, 514CB1a1 (5'-ATGACNATHGGNTGGGC-3') and 514CB2a2 (5'-RTANGCDATNGCNKNGCC-3'), were designed based on the circularin A amino acid sequences and two others, B574-for4 (5'-GGNWSNGGNYTNTT YGGNGGNGC-3') and B574-rev1 (5'-GCNCKYTTNCDATYTTNGTCCA-3'), were designed based on the clostin B574 amino acid sequence. DNA fragments obtained after PCR with these primers on the appropriate chromosomal DNA templates were cloned by using the Zero-Blunt TOPO PCR cloning kit (Invitrogen, Breda, The Netherlands) or in pUC19 digested with *Sma*I. PCR products were purified by using the High Pure PCR product purification kit from Roche. Sequencing was performed with the ALFII system with Cy5-labeled universal and reverse primers according to the protocols of the supplier (Amersham) with the following modifications: the power for the long-read gels was set at 15 W and the power for the high-resolution gels was set at 18 W.

**Computational analyses.** Homology comparisons were performed by using the basic logical alignment tool (BLAST) as described by Altschul et al. (1). BLAST searches were performed against the National Center for Biotechnology Information nonredundant protein database and the National Center for Biotechnology Information microbial genome database. Signal peptides were identified by using signalP (46). Putative transmembrane helices were identified with the HMTMM2.0 program (37). Proteinase degradation of bacteriocins was predicted by the PeptideCutter program, available at the ExPASy molecular biology server. Dyad symmetry, isoelectric point (pI), and molecular weight were determined by using the program Clonemanager 4 (SEcentral; Scientific & Educational Software).

**Nucleotide sequence accession number.** The circularin A sequence is available under GenBank accession no. AY164463, and the clostin 574 sequence is available under GenBank accession no. AY164462.

## RESULTS

**Bacteriocin production by clostridia.** Twelve clostridial strains were tested for their ability to inhibit *C. tyrobutyricum* B570, a known cheese spoilage bacterium (36). Two of these strains, *C. beijerinckii* ATCC 25752 and *C. tyrobutyricum* ADRIAT 932 exhibited antibacterial activity towards the indicator strain. The antibacterial activities produced by the *C. beijerinckii* strain and the *C. tyrobutyricum* strain were named circularin A and clostin 574, respectively, based on the results

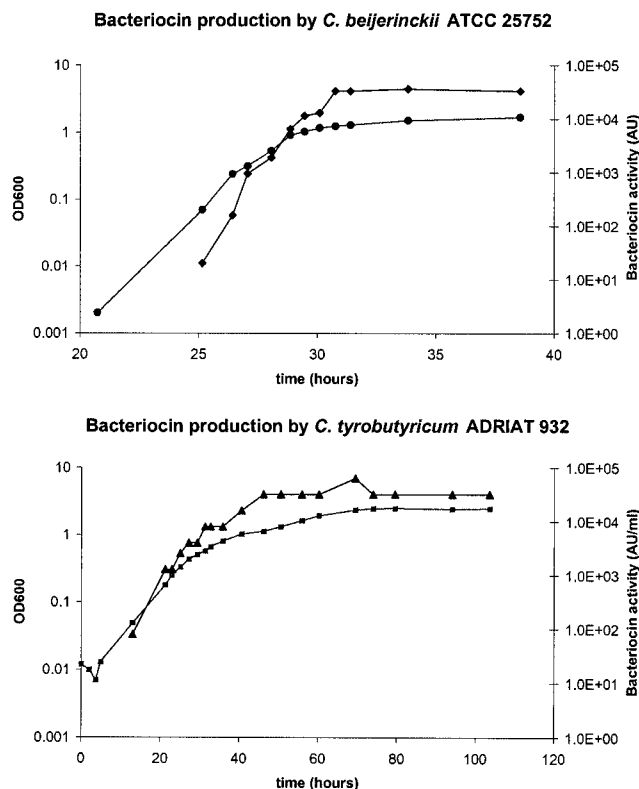


FIG. 1. Bacteriocin production by *C. beijerinckii* ATCC 25752 and *C. tyrobutyricum* ADRIAT 932. Bacterial growth, followed by measurement of the optical density of the cultures at 600 nm, is indicated by circles and squares, respectively, while bacteriocin activity is shown by diamonds and triangles, respectively. The optical densities at 600 nm (OD600) are plotted on the left y axes, while the right y axes show the bacteriocin activities (in AU per milliliter) in both culture supernatants.

presented below and were further characterized. Production of the antibacterial activities was shown to take place in the exponential growth phase of each strain although clostin 574 production seems to be completed before the culture enters the stationary phase. (Fig. 1). The treatment of culture supernatants with proteinases caused complete loss of clostin 574 activity while circularin A activity was more resistant to proteinase digestion. Circularin A activity was only completely lost when the supernatant of producing cells was treated with proteinase K (1.0 mg/ml), whereas treatment with other proteinases such as thermolysin (1.0 mg/ml), pronase (1.0 mg/ml), trypsin (0.78 mg/ml), and alpha-chymotrypsin (1.0 mg/ml) resulted in residual antibacterial activity (2 to 100%) even after 24 h of incubation. Samples of circularin A-containing supernatant after proteinase digestion were analyzed by a gel overlay assay. The band of inhibition of growth of the indicator had not shifted, indicating that digestion products of circularin A are not the cause of the residual antibacterial activity (data not shown). These results are indicative of the proteinaceous nature of both antibacterial activities. Incubation at 80 or 90°C for 1 h causes complete inactivation of both bacteriocins. Incubation at somewhat less-elevated temperatures (between 50 and 80°C) causes a significant reduction in clostin 574 activity while circularin A activity is stable at these temperatures. The



TABLE 2. Activity spectrum of circularin A and closticin 574

Indicator strain	Result <sup>a</sup> for:	
	Circularin A	Closticin 574
<i>C. beijerinckii</i> ATCC 25752	—	+
<i>C. tyrobutyricum</i> NIZO B570	+	+
<i>C. tyrobutyricum</i> ADRIAT 932	+	—
<i>C. tyrobutyricum</i> NIZO B575	+	+
<i>C. tyrobutyricum</i> CNRZ500	+	+
<i>C. tyrobutyricum</i> NIZO B590	+	+
<i>E. faecalis</i> JH2-2	+	—
<i>E. faecalis</i> OG1X	+	—
<i>Lactobacillus alimentarius</i> L4	+	+
<i>Lactobacillus brevis</i> L40	—	—
<i>Lactobacillus buchneri</i> L4	—	+
<i>Lactobacillus casei</i> subsp. <i>casei</i> L37	—	—
<i>Lactobacillus plantarum</i> lacC	—	—
<i>Lactobacillus sake</i> ATCC 15521	+	+
<i>Lactobacillus sake</i> IFO12456	+	—
<i>L. lactis</i> IL1403	+	—
<i>L. lactis</i> MG1363	+	—
<i>Pediococcus pentosaceus</i> FBB61-2	—	—
<i>Pediococcus pentosaceus</i> PPE 1.2	—	—

<sup>a</sup> +, inhibition; —, no inhibition of the indicator strain by 5×-diluted culture supernatant of *C. beijerinckii* ATCC 25752 (circularin A producer) or *C. tyrobutyricum* ADRIAT 932 (closticin 574 producer) in a microtiter plate assay.

bacteriocins do not seem to be extremely oxygen sensitive, as both inhibit *L. sake* ATCC 15521 in the presence of oxygen. All of these properties define both antibacterial activities as bacteriocins. DTT did not destroy the activity of either bacteriocin (data not shown), indicating that both probably do not contain the disulfide bridges necessary for antibacterial activity.

The activity spectrum of both bacteriocins was determined (Table 2). Circularin A has the widest activity range, inhibiting all *C. tyrobutyricum* strains tested and also lactococci, enterococci, and some *Lactobacillus* strains. Closticin 574 also inhibits all *C. tyrobutyricum* strains tested, except for the producer strain, and some lactobacilli but not the enterococci and *Lactococci* tested. Circularin A and closticin 574 are both small proteins. The supernatant of *C. beijerinckii* ATCC 25752 produced a band of inhibition at a position of a protein of 2.2 kDa in a gel overlay assay (Fig. 2). The supernatant of the closticin

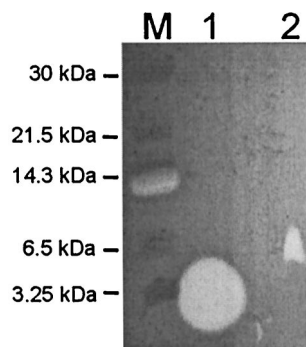


FIG. 2. Circularin A and closticin 574 activity in a Tricine-SDS-PAGE gel overlay assay. Lanes: 1, 0.125  $\mu$ l of *C. beijerinckii* ATCC 25752 supernatant; 2, 7.5  $\mu$ l of *C. tyrobutyricum* ADRIAT 932 supernatant; M, 4  $\mu$ l of Rainbow molecular weight marker RPN 755. The band of inhibition in lane M corresponds to lysozyme activity. The indicator strain used was *L. sake* ATCC 15521.

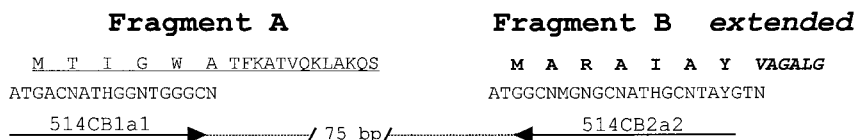
574 producer gave a growth-inhibition halo at the position of a protein of approximately 7 kDa (Fig. 2). When 2× Laemmli sample buffer with SDS and  $\beta$ -mercaptoethanol was used for sample preparation, no activity of closticin 574 was observed in the gel overlay assay. Omitting SDS and  $\beta$ -mercaptoethanol solved this problem. Circularin A was not affected by these constituents of the sample buffer. These results indicate that closticin 574, unlike circularin A, is sensitive to denaturing conditions.

**Circularin A purification.** The initial purification of circularin A was performed by using nondialyzed complete AC medium. However, impurity of the bacteriocin preparation with medium peptides precluded proper determination of the N-terminal amino acid sequence of circularin A. The use of dialyzed medium (see Materials and Methods) solved this problem. Production in dialyzed medium was between 8- to 38-fold lower than production in complete medium. The total bacteriocin activity in a stationary-phase culture grown in dialyzed medium was approximately  $6.4 \times 10^4$  AU/ml. Using 50% ammonium sulfate, 31% of the activity could be precipitated. The precipitate was dissolved in demineralized water and extracted with *n*-butanol. No antibacterial activity was lost during this step of the isolation procedure. The *n*-butanol fraction was lyophilized, the residue was dissolved in water, and the sample was subjected to HPLC purification. Bacteriocin activity eluted in two peaks of which the first had the highest antibacterial activity (50 to 70% of the total activity applied). The second peak had less than 1% of the antibacterial activity of the first peak. Whether this peak is related to circularin A or is caused by another bacteriocin is unknown. Because of the low level of bacteriocin activity it was not further studied. The purified protein in the first peak was judged pure by Tricine-SDS-PAGE and silver staining, and the amount was estimated to be approximately 100 pmol (data not shown).

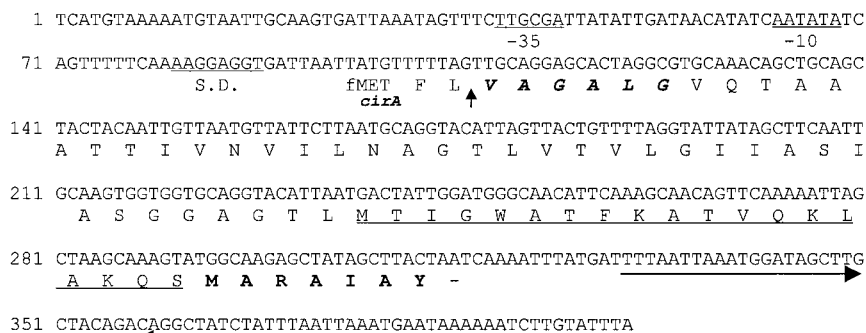
**Internal amino acid sequence of circularin A and identification of the *cirA* structural gene by reverse genetics.** Initial attempts to determine the N-terminal amino acid sequence of purified circularin A failed, indicating that the N terminus may be blocked. After cyanogen bromide (CnBr) cleavage of the protein, a mixture of two signals was obtained during N-terminal sequencing. This result indicated that the protein was cut into two fragments and contained at least two methionine residues. The mixture of fragments (A and B) after CnBr cleavage was purified by HPLC-C<sub>18</sub>, and the N-terminal amino acid sequence of fragment A was determined (Fig. 3). Combining the amino acid sequence of this fragment with the N-terminal amino acid sequence determined on the fragment mixture obtained after CnBr cleavage enabled deduction of the amino acid sequence of fragment B (Fig. 3).

In *C. beijerinckii* ATCC 25752, no plasmids could be detected (data not shown), and therefore, the genes for bacteriocin production are chromosomally located. Based on the amino acid sequences of the two fragments A and B, a number of degenerate primers were designed and used in different combinations as the relative orientation of fragments A and B was a priori unknown. A 75-bp PCR fragment was obtained from chromosomal DNA from *C. beijerinckii* ATCC 25752 with primers 514CB1a1 and 514CB2a2. This result indicated that the fragments A and B occur in the order A to B in the bacteriocin (Fig. 3). The PCR fragment was subcloned in

A



B



C



- 1) Proteolytic cleavage L<sup>3</sup>-V<sup>4</sup>
- 2) Peptide bond formation Y<sup>72</sup>-V<sup>4</sup>

FIG. 3. Nucleotide sequence of *cirA* and circularization of circularin A. The fragments A (underlined), B (boldface), and B extended (boldface and italic) are indicated throughout the figure. (A) Amino acid sequences of fragments A and B of CnBr-cleaved circularin A. The sequence of fragment A was obtained by Edman degradation on a purified fragment of CnBr-cleaved circularin A. The sequence of fragment B was obtained by deduction as described in the text. The extended sequence of fragment B was used to determine the circularization point. The degenerate nucleotide sequences of the primers (514CB1a1 and 514CB2a2) used to obtain an internal *cirA* DNA fragment by PCR (dotted line) are indicated. (B) Nucleotide and deduced amino acid sequences of the structural gene of circularin A (*cirA*). The vertical arrow indicates the circularization point. Putative Shine-Dalgarno (S.D.) and promoter sequences are underlined. Translation starts with a formyl-methionine (fMet). Two large arrows indicate an inverted repeat downstream of *cirA*. (C) Putative maturation process of circularin A. The peptide bond between Leu<sup>3</sup> and Val<sup>4</sup> is cleaved, and a new bond is formed between Tyr<sup>72</sup> and Val<sup>4</sup>. The reaction yields the peptide MFL and the circular bacteriocin. The thin line in the pre-CirA sequence is a schematic representation of the newly forming peptide bond.

pUC19 and sequenced. The entire structural gene of circularin A was subsequently cloned by inverse PCR techniques and sequenced (Fig. 3). The sequence is available under GenBank accession no. AY164463. *CirA* codes for a peptide of 72 amino acid residues. The gene is preceded by a proper Shine-Dal-

garno sequence (AAGGAGGT) at a distance of 6 nucleotides upstream of the translation initiation codon. Putative -35 (TTGCGA) and -10 (TATCAA) sequences are present. A large palindromic structure ( $\Delta G_0 = -20.7$  kcal) is present downstream of *cirA*. Performing a BLAST search with the derived

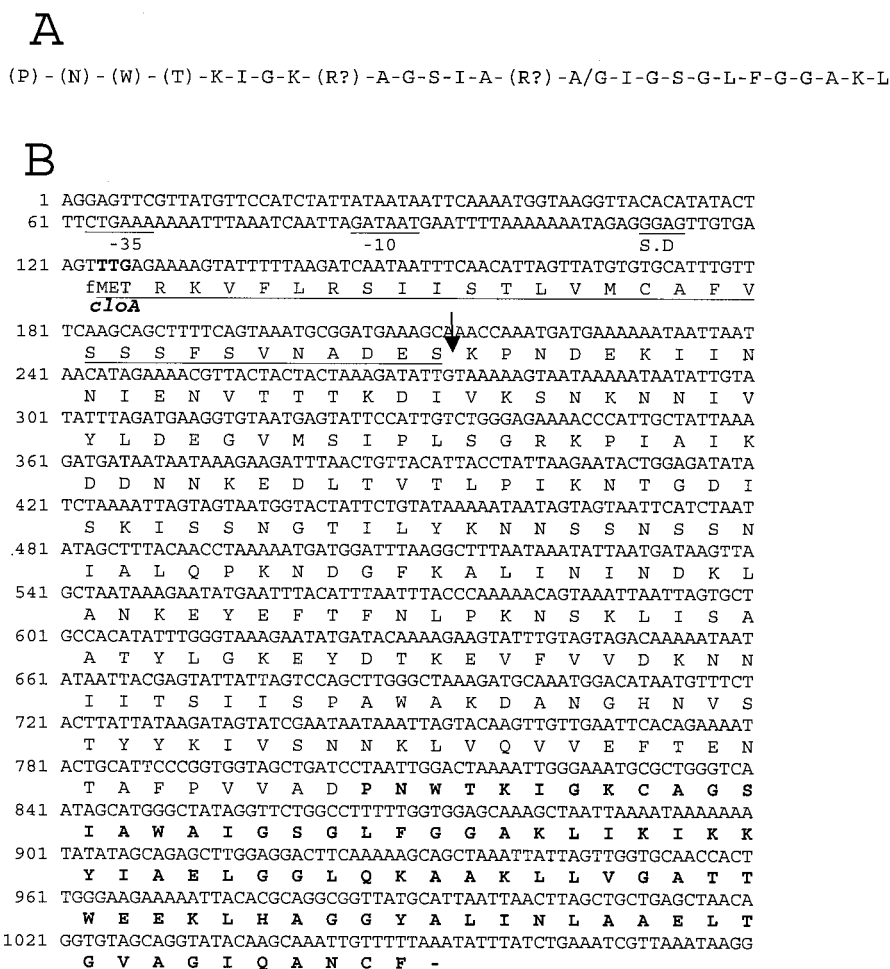


FIG. 4. Nucleotide and derived amino acid sequences of closticin 574. (A) N-terminal amino acid sequence of closticin 574 as determined by Edman degradation on the purified protein. Amino acids within parentheses were determined with a confidence level of >90% certainty. Parenthetic amino acids with a question mark obtained a confidence level of 50%. At position 16, signals of both Ala and Gly were derived, probably as a consequence of high background levels of glycine. (B) Nucleotide and deduced amino acid sequences of the structural gene of closticin 574 (*cloA*). The deduced amino acid sequence of mature closticin 574 is indicated in boldface. The predicted N-terminal signal sequence is underlined. Putative Shine Dalgarno (S.D) and promoter sequences are underlined. The putative UUG start codon is indicated in boldface.

circularin A amino acid sequence revealed that the protein has 60% similarity (30% identity) with the *E. faecalis* bacteriocin AS-48 (40). AS-48 is a circular molecule, and the purified peptide was also blocked at the N terminus (40). Continuing the N-terminal amino acid sequence reactions on the CnBr-cleaved fragment mixture, the sequence of fragment B was extended. In this way it was shown that circularin A is also a circular peptide. It also allowed determination of the exact point of circularization (Fig. 3). Mature circularin A contains 69 amino acids. It is formed by the removal of a leader of three amino acid residues from the preprotein and head-to-tail ligation between the processed N terminus and the C terminus. Circularin A has high pI of 10.60 as calculated on the noncircular form of the mature bacteriocin, which is common for bacteriocins.

**Purification of closticin 574.** The supernatant of a producing culture of *C. tyrobutyricum* ADRIAT 932 containing  $6.4 \times 10^7$  AU of bacteriocin activity was precipitated with 40% ammonium sulfate. In this way, 80% of the total activity was recov-

ered. The precipitate, dissolved in 20 ml of 50 mM phosphate buffer (pH 7.0) with 30% isopropanol and 1.0 mM ammonium sulfate, was purified by FPLC with an ammonium sulfate gradient (see Materials and Methods). Bacteriocin activity eluted around 300 mM ammonium sulfate with 30% isopropanol, and the recovery of activity after the FPLC step was 100%. The pooled active fractions were concentrated by ultrafiltration and DEAE-column chromatography applying a NaCl gradient. Of the total activity, 35% was present in the flowthrough while 18% of the activity eluted between 1.6 and 2.0 M NaCl. The loss of bacteriocin activity could be caused by degradation and/or chemical modification of the bacteriocin or could indicate that the total bacteriocin activity is based on more than one protein. The flowthrough was concentrated by column ultrafiltration and subjected to HPLC. The activity retrieved after this step was only about 20% of that applied to the column. The quantity (10 to 20 pmol) and purity were sufficient, as judged by Tricine-SDS-PAGE and silver staining, to allow for N-terminal amino acid sequence determination by Edman degradation.

**N-terminal amino acid sequence of closticin 574 and isolation of the *cloA* structural gene.** The 27 N-terminal amino acid residues of purified closticin 574 were determined (Fig. 4). Degenerate primers B574-for4 and B574-rev1 were designed based on this sequence and used for inverse PCR on *Sau*3A-digested and self-ligated *C. tyrobutyricum* ADRIAT 932 chromosomal DNA. A 265-bp PCR fragment was obtained, cloned in the pCR-Blunt II-TOPO vector, and sequenced. Based on the obtained sequence, primers were designed and used for further PCR and sequencing. The nucleotide sequence of the structural gene of closticin 574 (*cloA*) is presented in Fig. 4. The sequence is available under GenBank accession no. AY164462. From the nucleotide and N-terminal amino acid sequence data, closticin 574 is concluded to be initially produced as a preproprotein of 310 amino acid residues. It is subsequently processed to a bacteriocin of 82 amino acids with a pI of 9.9. The protein has an unusually long preproprotein sequence of 228 amino acids. Translation initiation putatively starts at a rare UUG start codon. A putative ribosome binding site (GGAG) is present 5 nucleotides upstream of this start codon, and the *cloA* gene is preceded by putative  $-35$  (CTG AAA) and  $-10$  (GATAAT) sequences. The deduced amino acid sequence of *cloA* is almost identical to the sequence obtained by Edman degradation. Arg<sup>9</sup> and Arg<sup>15</sup> in the Edman degradation profile are actually cysteine and tryptophan residues, respectively, in the deduced peptide. CloA is homologous to the deduced products of two unknown open reading frames of *Corynebacterium diphtheriae* (<http://www.sanger.ac.uk>) and to one unknown product of *Corynebacterium glutamicum* (accession no. AX127147). The first 27 amino acids of the primary translation product of *cloA* were identified as a signal sequence by the SignalP program (Fig. 4).

## DISCUSSION

Closticin 574 is, on the basis of the nucleotide sequence of its structural gene, produced as preproprotein of 309 amino acid residues. After secretion and processing, it gives rise to an antimicrobial peptide of 82 amino acid residues. The first 27 amino acids of the full-length protein probably constitute a general signal peptide, as predicted by the SignalP program. General signal peptides are uncommon for bacteriocins, although some bacteriocins, such as enterocin P, divergicin A, and listeriocin (9, 30, 61), are secreted via the general secretion pathway. Furthermore, secretion of a bacteriocin that normally is externalized by dedicated, *sec*-independent secretion systems can be accomplished by fusing it to a signal peptide directing the heterologous protein to the general secretion pathway (5, 42). The predicted secretion of CloA via the *sec* pathway and subsequent processing of the putative signal peptide would not yield the bioactive peptide. The secreted protein is most probably further processed extracellularly either by a general or by a specific proteinase. Such a mechanism of bacteriocin activation has been observed before. The bacteriocin Lsb1 of *Lactococcus lactis*, for instance, has been recently shown to be processed, and thus activated, after secretion by the cell wall proteinase HtrA (16a). It is also possible that the aspartyl-prolyl bond (Asp<sup>227</sup>-Pro<sup>228</sup>) at which cleavage in closticin 574 has taken place is chemically broken to create the active peptide, as is the case for guanylin (54). However, despite the fact

that the N-terminally sequenced protein was active, we cannot exclude the possibility that the cleavage of the Asp<sup>227</sup>-Pro<sup>228</sup> bond is the result of the purification method, as Asp-Pro bonds are easily hydrolyzed under acidic conditions (38, 48). During purification of closticin 574, such acidic conditions are encountered in the HPLC step when the sample is eluted in the presence of trifluoroacetic acid, a very acidic compound. Cleavage of Asp-Pro bonds during protein purification has been observed previously (16, 54, 55, 57).

The cysteines present in closticin 574 are not involved in the formation of a disulfide bridge required for activity since DTT treatment did not inactivate the protein. With all data taken together, closticin A classifies as a class IIc bacteriocin, although it is exceptional for its unusual maturation process. The classification is based on its sensitivity for proteinases and the relatively small size (82 amino acid residues) (35) of the processed prepeptide. Closticin A is not structurally modified like the class I bacteriocins (51), since none of the characteristic residues and problems typical when sequencing proteins contain lanthionine,  $\beta$ -methyllanthionine, or dehydroalanine residues (51) were encountered during N-terminal sequencing. The bacteriocin does not contain the YGNGVXAAC consensus motif of the pediocin-like bacteriocins (15), nor are two different peptides required for activity, as is the case for lactococcin G, lactococcin M/N, and plantaricins EF and JK (3, 47, 61).

Circularin A is produced as a preprotein of 72 amino acids that is processed to a circular peptide of 69 amino acid residues. The preprotein is circularized by the hydrolysis of the peptide bond between Leu<sup>3</sup> and Val<sup>4</sup> and the formation of a new bond between Val<sup>4</sup> and the C-terminal Tyr<sup>72</sup> (Fig. 3). This head-to-tail linkage has so far only been described for three other proteins, gassericin A, microcin J25, and AS-48, all of which are antibacterial peptides (6, 31, 40). The circularization of microcin J25 is accomplished by two dedicated proteins (56). For AS-48, it is not known which proteins, out of four different proteins involved in the production of the active bacteriocin, are responsible for the actual circularization (41). The actual location at which circularization takes place is not known for any of the four bacteriocins. The homology between the two systems for production of microcin J25 or AS-48 is limited to the presence of an ATP-binding site in one of the proteins involved in each case. Circularin A is similar to AS-48 but, interestingly, lacks the extended leader of AS-48 (40). Only the first three N-terminal amino acids of pre-CirA are cleaved off to allow the formation of a peptide bond between Val<sup>4</sup> and Tyr<sup>72</sup>. The three other circular bacteriocins are translated with longer leader peptides of 33, 35, and 37 residues for gassericin A, AS-48, and microcin J25, respectively (6, 31, 40). The question arises as to whether the 3-amino-acid-long leader in pre-CirA has a function in targeting the protein for secretion or processing, as do the signal peptides and the double glycine leaders of other bacteriocins (23, 60). Some bacteriocins are known to be secreted without the need for a leader peptide (9, 16a, 45), but these bacteriocins are not modified nor is the transport machinery dedicated to that bacteriocin. It has been postulated that in those cases the overall charge is the recognizing factor (16a). Another possibility might be that the signal directing the protein for modification and secretion is embedded within the peptide, as has been shown for hemolysin (34),



some metalloproteinases (21), and some peroxisomal proteins (58). That a few amino acid residues can already have a targeting function has been shown for the peroxisome-targeting signals PTS1 and PTS2. PTS1 is a conserved C-terminal tripeptide sequence while PTS2 is a nonapeptide sequence located near the N terminus or at internal locations of the protein to be targeted (58). If the 3-amino-acid residue leader peptide of pre-circularin A embeds a targeting function, it would be the smallest leader peptide known.

Circularin A is highly resistant to digestion by sequence-specific endoproteases. This is not due to absence of digestion sites, since sites for trypsin (Lys-X, Arg-X), alpha-chymotrypsin (FYW-X [not P,M]), and thermolysin (X [not D,E]-A, F, I, L, M, V) are present in circularin A. This same resistance to proteolytic digestion has been observed for microcin J25 (6). AS-48 was resistant to digestion with alpha-chymotrypsin (29) but was hydrolyzed by trypsin, pronase, and proteinase K (17). We presume that the resistance to proteolytic hydrolysis is due to the inaccessibility of the recognition sites. This notion seems to be supported by the three-dimensional structure of the circularin A homologue AS-48. AS-48 is a tightly folded globular peptide consisting of 5  $\alpha$ -helices, resembling the typical saporin fold as observed in NK-lysin, a porcine antibacterial peptide from NK and T cells (22). The functionality of such resistance towards proteinases could be to make the protein less susceptible to digestion by proteinases of target bacteria, thereby increasing the activity range of the bacteriocin. For instance, the natural cell wall proteinases PrtPI and PrtPIII of *L. lactis* digest bacteriocins such as LcnB, LcnA, and Lsb2 (16a) but are unable to digest circularin A (unpublished data).

Classification of the circular bacteriocins circularin A, microcin J25, gasserin A, and AS-48 in either of the current bacteriocin classes I to III is not justifiable. They are ribosomally synthesized and posttranslationally modified small bacteriocins, which makes them fall out of bacteriocin classes II and III. Due to the fact that they do not contain modified amino acids, they do not belong to the class I bacteriocins, and since they are not complex proteins composed of a protein plus another chemical moiety (lipid or carbohydrate) needed for activity, they cannot be in class IV. We therefore propose to distinguish a new class, class V, of bacteriocins consisting of ribosomally synthesized nonmodified head-to-tail-ligated cyclic antibacterial peptides. Characteristic of class V bacteriocins is their cyclization by formation of a peptide bond between the N and C termini of a processed prepeptide. This process of leader peptide removal, circularization, and secretion putatively involves dedicated enzymatic reactions. These enzymatic reactions are most probably encoded by the genes in the direct vicinity of the structural gene (unpublished data).

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#### REFERENCES

- Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389–3402.
- Anderson, D. G., and L. L. McKay. 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl. Environ. Microbiol.* **46**:549–552.
- Anderssen, E. L., D. B. Diep, I. F. Nes, V. G. Eijsink, and J. Nissen-Meyer. 1998. Antagonistic activity of *Lactobacillus plantarum* C11: two new two-peptide bacteriocins, plantaricins EF and JK, and the induction factor plantaricin A. *Appl. Environ. Microbiol.* **64**:2269–2272.
- Andersson, M., H. Gunne, B. Agerberth, A. Boman, T. Bergman, R. Sillard, H. Jornvall, V. Mutt, B. Olsson, H. Wigzell et al. 1995. NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. *EMBO J.* **14**:1615–1625.
- Biet, F., J. M. Berjeaud, R. W. Worobo, Y. Cenatiempo, and C. Fremaux. 1998. Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway. *Microbiology* **144**:2845–2854.
- Blond, A., J. Peduzzi, C. Goulard, M. J. Chiuchiole, M. Barthelemy, Y. Prigent, R. A. Salomon, R. N. Farias, F. Moreno, and S. Rebuffat. 1999. The cyclic structure of microcin J25, a 21-residue peptide antibiotic from *Escherichia coli*. *Eur. J. Biochem.* **259**:747–755.
- Buhnia, A. K., M. C. Johnson, and B. Ray. 1987. Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate polyacrylamide gel electrophoresis. *J. Indust. Microbiol.* **2**:319–322.
- Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella. 1984. Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. *Plasmid* **11**:260–263.
- Cintas, L. M., P. Casaus, L. S. Håvarstein, P. E. Hernandez, and I. F. Nes. 1997. Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. *Appl. Environ. Microbiol.* **63**:4321–4330.
- Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* **71**:1–20.
- Danders, W., M. A. Marahiel, M. Krause, N. Kosui, T. Kato, N. Izumiya, and H. Kleinkauf. 1982. Antibacterial action of gramicidin S and tyrocidines in relation to active transport, in vitro transcription, and spore outgrowth. *Antimicrob. Agents Chemother.* **22**:785–790.
- Dineen, S. S., M. Bradshaw, and E. A. Johnson. 2000. Cloning, nucleotide sequence, and expression of the gene encoding the bacteriocin botocin B from *Clostridium botulinum* strain 213B. *Appl. Environ. Microbiol.* **66**:5480–5483.
- Duitman, E. H., L. W. Hamoen, M. Rembold, G. Venema, H. Seitz, W. Saenger, F. Bernhardt, R. Reinhardt, M. Schmidt, C. Ullrich, T. Stein, F. Leenders, and J. Vater. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proc. Natl. Acad. Sci. USA* **96**:13294–13299.
- Edman, P. 1956. On the mechanism of the phenyl isothiocyanate degradation of peptides. *Acta Chem. Scand.* **10**:761–768.
- Ennahar, S., T. Sashihara, K. Sonomoto, and A. Ishizaki. 2000. Class IIA bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol. Rev.* **24**:85–106.
- Fairchild, T. A., D. Fulton, J. T. Fontana, J. P. Gratton, T. J. McCabe, and W. C. Sessa. 2001. Acidic hydrolysis as a mechanism for the cleavage of the Glu(298)→Asp variant of human endothelial nitric-oxide synthase. *J. Biol. Chem.* **276**:26674–26679.
- Gajic, O. 2002. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
- Galvez, A., M. Maqueda, E. Valdivia, A. Quesada, and E. Montoya. 1986. Characterization and partial purification of a broad spectrum antibiotic AS-48 produced by *Streptococcus faecalis*. *Can. J. Microbiol.* **32**:765–771.
- Garnier, T., and S. T. Cole. 1986. Characterization of a bacteriocinogenic plasmid from *Clostridium perfringens* and molecular genetic analysis of the bacteriocin-encoding gene. *J. Bacteriol.* **168**:1189–1196.
- Gasson, M. J. 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J. Bacteriol.* **154**:1–9.
- Geis, A., J. Singh, and M. Teuber. 1983. Potential of lactic streptococci to produce bacteriocin. *Appl. Environ. Microbiol.* **45**:205–211.
- Ghigo, J. M., and C. Wandersman. 1992. Cloning, nucleotide sequence and characterization of the gene encoding the *Erwinia chrysanthemi* B374 PrtA metalloprotease: a third metalloprotease secreted via a C-terminal secretion signal. *Mol. Gen. Genet.* **236**:135–144.
- Gonzalez, C., G. M. Langdon, M. Bruix, A. Galvez, E. Valdivia, M. Maqueda, and M. Rico. 2000. Bacteriocin AS-48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin. *Proc. Natl. Acad. Sci. USA* **97**:11221–11226.
- Håvarstein, L. S., D. B. Diep, and I. F. Nes. 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Mol. Microbiol.* **16**:229–240.
- Hewick, R. M., M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer. 1981. A

- gas-liquid solid phase peptide and protein sequenator. *J. Biol. Chem.* **256**: 7990–7997.
25. Ike, Y., and D. B. Clewell. 1984. Genetic analysis of the pAD1 pheromone response in *Streptococcus faecalis*, using transposon Tn917 as an insertional mutagen. *J. Bacteriol.* **158**:777–783.
  26. Ilse, D., and P. Edman. 1963. The formation of 3-phenyl-2-thiohydantoins from phenylthiocarbonyl amino acids. *Aust. J. Chem.* **16**:411–416.
  27. Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev.* **59**:171–200.
  28. Jacob, A. E., and S. J. Hobbs. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. zymogenes. *J. Bacteriol.* **117**:360–372.
  29. Joosten, H. M., M. Nunez, B. Devreese, J. Van Beeumen, and J. D. Marugg. 1996. Purification and characterization of enterocin 4, a bacteriocin produced by *Enterococcus faecalis* INIA 4. *Appl. Environ. Microbiol.* **62**:4220–4223.
  30. Kalmokoff, M. L., S. K. Banerjee, T. Cyr, M. A. Hefford, and T. Gleeson. 2001. Identification of a new plasmid-encoded sec-dependent bacteriocin produced by *Listeria innocua* 743. *Appl. Environ. Microbiol.* **67**:4041–4047.
  31. Kawai, Y., T. Saito, H. Kitazawa, and T. Itoh. 1998. Gasserin A: an uncommon cyclic bacteriocin produced by *Lactobacillus gasserii* LA39 linked at N- and C-terminal ends. *Biosci. Biotechnol. Biochem.* **62**:2438–2440.
  32. Kawai, Y., T. Saito, M. Suzuki, and T. Itoh. 1998. Sequence analysis by cloning of the structural gene of gasserin A, a hydrophobic bacteriocin produced by *Lactobacillus gasserii* LA39. *Biosci. Biotechnol. Biochem.* **62**: 887–892.
  33. Keis, S., C. F. Bennett, V. K. Ward, and D. T. Jones. 1995. Taxonomy and phylogeny of industrial solvent-producing *clostridia*. *Int. J. Syst. Bacteriol.* **45**:693–705.
  34. Kenny, B., R. Haigh, and I. B. Holland. 1991. Analysis of the haemolysin transport process through the secretion from *Escherichia coli* of PCM, CAT or beta-galactosidase fused to the Hly C-terminal signal domain. *Mol. Microbiol.* **5**:2557–2568.
  35. Klaenhammer, T. R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev.* **12**:39–85.
  36. Klijn, N., F. F. Nieuwenhof, J. D. Hoolwerf, C. B. van der Waals, and A. H. Weerkamp. 1995. Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. *Appl. Environ. Microbiol.* **61**:2919–2924.
  37. Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**:567–580.
  38. Landon, M. 1977. Cleavage at aspartyl-prolyl bonds. *Methods Enzymol.* **47**:145–149.
  39. Mahony, D. E., R. Ahmed, and S. G. Jackson. 1992. Multiple typing techniques applied to a *Clostridium perfringens* food poisoning outbreak. *J. Appl. Bacteriol.* **72**:309–314.
  40. Martinez-Bueno, M., M. Maqueda, A. Galvez, B. Samyn, J. Van Beeumen, J. Coyette, and E. Valdivia. 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. *J. Bacteriol.* **176**:6334–6339.
  41. Martinez-Bueno, M., E. Valdivia, A. Galvez, J. Coyette, and M. Maqueda. 1998. Analysis of the gene cluster involved in production and immunity of the peptide antibiotic AS-48 in *Enterococcus faecalis*. *Mol. Microbiol.* **27**: 347–358.
  42. McCormick, J. K., R. W. Worobo, and M. E. Stiles. 1996. Expression of the antimicrobial peptide carnobacteriocin B2 by a signal peptide-dependent general secretory pathway. *Appl. Environ. Microbiol.* **62**:4095–4099.
  43. Mootz, H. D., and M. A. Marahiel. 1997. The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. *J. Bacteriol.* **179**:6843–6850.
  44. Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Leeuwenhoek* **70**:113–128.
  45. Netz, D. J., H. G. Sahl, R. Marcolino, N. J. dos Santos, S. S. de Oliveira, M. B. Soares, and M. C. Freire Bastos. 2001. Molecular characterisation of aureocin A70, a multi-peptide bacteriocin isolated from *Staphylococcus aureus*. *J. Mol. Biol.* **311**:939–949.
  46. Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**:1–6.
  47. Nissen-Meyer, J., H. Holo, L. S. Havarstein, K. Sletten, and I. F. Nes. 1992. A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *J. Bacteriol.* **174**:5686–5692.
  48. Piskiewicz, D., M. Landon, and E. L. Smith. 1970. Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. *Biochem. Biophys. Res. Commun.* **40**:1173–1178.
  49. Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenberg. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Appl. Environ. Microbiol.* **54**:2349–2353.
  50. Rood, J. L., and S. T. Cole. 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiol. Rev.* **55**:621–648.
  51. Sahl, H. G., and G. Bierbaum. 1998. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu. Rev. Microbiol.* **52**:41–79.
  52. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  53. Schagger, H., and G. Von Jagow. 1987. Tricin-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**:368–379.
  54. Schulz, A., U. C. Marx, Y. Hidaka, Y. Shimonishi, P. Rosch, W. G. Forssmann, and K. Adermann. 1999. Role of the prosequence of guanylin. *Protein Sci.* **8**:1850–1859.
  55. Segalas, I., R. Thai, R. Menez, and C. Vita. 1995. A particularly labile Asp-Pro bond in the green mamba muscarinic toxin MTX2. Effect of protein conformation on the rate of cleavage. *FEBS Lett.* **371**:171–175.
  56. Solbiati, J. O., M. Ciaccio, R. N. Farias, J. E. Gonzalez-Pastor, F. Moreno, and R. A. Salomon. 1999. Sequence analysis of the four plasmid genes required to produce the circular peptide antibiotic microcin J25. *J. Bacteriol.* **181**:2659–2662.
  57. Storic, P., A. Tossi, B. Lenarcic, and D. Romeo. 1996. Purification and structural characterization of bovine cathelicidins, precursors of antimicrobial peptides. *Eur. J. Biochem.* **238**:769–776.
  58. Subramani, S., A. Koller, and W. B. Snyder. 2000. Import of peroxisomal matrix and membrane proteins. *Annu. Rev. Biochem.* **69**:399–418.
  59. Tomita, H., S. Fujimoto, K. Tanimoto, and Y. Ike. 1997. Cloning and genetic and sequence analyses of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. *J. Bacteriol.* **179**:7843–7855.
  60. Van Belkum, M. J., R. W. Worobo, and M. E. Stiles. 1997. Double-glycine-type leader peptides direct secretion of bacteriocins by ABC transporters: colicin V secretion in *Lactococcus lactis*. *Mol. Microbiol.* **23**:1293–1301.
  61. Worobo, R. W., M. J. Van Belkum, M. Sailer, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1995. A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. *J. Bacteriol.* **177**:3143–3149.
  62. Wray, W., T. Boulukas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203.